A

Ph. D. RESEARCH SYNOPSIS ON

"SCREENING AND ISOLATION OF NATURALLY OCCURRING MICROBIAL PIGMENTS, THEIR CHARACTERIZATION AND APPLICATIONS"

SUBMITTED BY

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INTRODUCTION

Man has been using chemical or synthetic colorants in foodstuff, cosmetic, textile and pharmaceutical manufacturing processes since ages. Due to such extensive use with proven side effects, has ultimately lead to use of pigments extracted from either plant or microbial source (Kang *et al.*, 1996). There is worldwide interest in bio-process development for the production of pigments from natural sources due to a serious safety problem with many artificial synthetic colorants, which have widely been used in foodstuff, cosmetic and pharmaceutical manufacturing processes (Kim *et al.*, 1995).

Colorants are used in a vast majority of products that humans use, ranging from food to clothing and textiles. However, a problem with many of these colorants is that they are synthetic, making them harmful to both living organism and environment because they are difficult to biodegrade. Furthermore, many synthetic dyes contain toxins that are harmful to humans. There has been a growing demand for natural dyes, which are biodegradable and have a less harmful impact on the environment. Natural dyes have better biodegradability and generally have higher compatibility with the environment. They are nontoxic, non-allergic to skin, non-carcinogenic, easily available (Adeel *et al.*, 2009; Pruthi *et al.*, 2007; Siva, 2007).

Pigmentation is a very common characteristic exhibited by many species of bacteria. Pigments are light-absorbing compounds that are responsible for the colors that organisms display. Pigment production varies greatly with the species and cultivation conditions (Cesar *et al*., 2005). Natural pigments like carotenoids, anthoquinone and chlorophyll are isolated from plants, animals and microorganisms such as algae, fungi and bacteria. The existing authorized natural food colorants are of either plant or animal origin and have numerous drawbacks such as instability against light, heat or adverse pH and low water solubility. Bacteria are considered as a good source of pigments. As they are originated from living organisms, they are also reffered as “Biocolours”. Plant and animal origin pigments are also available in nature but due to some drawbacks like availability throughout the year, its solubility in water, solubility in organic-inorganic solvents, sensitivity against heat and light, bacterial pigments are mostly preferred. Generally, aerobic microorganism produce pigments. Biocolours are identified as their secondary metabolic products. They have good potential for various industrial applications.

Dyes are generally chemically synthesized and available in large number whereas pigments are derived majority from natural sources and available in limited number. Pigments need a binding agent for gluing on fibre.

Microbial pigments are secondary metabolites, which are produced during stationary or late log phase by a variety of species. There are number of micro-organisms which have the ability to produce pigments in high yields such as species of genus *Monascus*, *Paecilomyces, Serratia*, *Cordyceps*, *Streptomyces* and *Penicillium.* Natural pigments do not have side effects. There is a great demand for microbial pigments due to their natural characters, safety to use, medicinal properties and easy production. Microorganisms can be directed to grow in laboratory in large numbers using waste materials and biomass obtained can be further subjected to treatment for extraction of pigments. Therefore, it is advantageous to produce natural pigments from microorganisms by using waste material to make process economical. The red and purple pigments extracted from *Serratia marceseus* and *Chryseobacterium sp.* known as prodigiosin and violacein respectively, have been used as colorants on different fabrics like acrylic fiber, silk, cotton, polyester, and polyester microfiber and their colorfastness on the fabrics was recorded by Ahmad *et al.*, 2012.

For industrial applications of microbial pigments, higher production of pigment yield, chemical and light stability are essential features. Isolation of new strain is still of particular interest because of necessity to obtain microorganisms with suitable characteristics. Certain problems with the use of natural dyes in textile dyeing are color yield, complexibility of dyeing process, reproducibility results, limited shades, blending problems and inadequate fastness properties (Sachan and Kapoor, 2007; Siva, 2007). But these problems can be overcome by using mordants which increase the affinity between fabric and the dye (Vankar *et al.*, 2009; Samanta and Agarwal, 2009).

One special property of natural dye which is not shared with synthetic dye is that they can absorb U.V. light and offer protection for skin from the harmful effects of Sun.

There are several advantages and disadvantages of natural colourant extracted from natural source over synthetic dye:

ADVANTAGES:

Utilizing natural pigment to impart colour on fabric has a number of advantages over synthetic dyes.

- Natural pigments are more eco-friendly than synthetic dyes. Synthetic dyeing procedures can be polluting and certain diazo dyes are carcinogenetic.
- Clothes dyed with natural pigment have the potential to be sold at a higher price.
- Depending on the dyeing procedure and the type of mordant used, a variety of colours can be produced using one natural dye source. Each natural dye source provides an amazing diversity of shades. From one dye you may obtain between 5-15 varying colours and shades.
- Inconsistencies in colour could be marketed as unique or one of a kind.

DISADVANTAGES:

The quality and effectiveness of natural pigment stuffs depends upon a great many factors. And, although there is a trend toward eco-friendly processes, natural pigments have some disadvantages.

- It takes time to extract the colour from raw materials.
- Natural pigments have limited availability.
- Although natural pigments initially produce bright colours in a variety of shades, fabrics tend to fade quicker than fabrics coloured with synthetic dyes when exposed to light and home laundering.
- Consistency of colour is a challenge when dyeing with natural pigment.
- Some mordants may present unacceptable levels of toxicity. The more toxic mordants, such as chromium and tin, are required for some of the brighter colours. It is important to note that not all mordants are toxic, for example alum, lime, turpentine, soya milk, banana flower, linseed oil is relatively safe to use, though not entirely non-toxic.
- Synthetic fibres usually cannot be dyed with natural dyes.

The present investigation reports the isolation, identification and characterization of pigments extracted from *Leifsonia aquatica*, *Pseudomonas aeruginosa*, *Kocuria rosea* and *Serratia marcescens* for effective application in textile industries and any other related dyeing area to promote natural pigment instead of synthetic mix. *Leifsonia aquatica*, *Pseudomonas aeruginosa*, *Kocuria rosea* and *Serratia marcescens* were isolated for yellow, green, orange and red colour pigment respectively from natural source and highest colour production was achieved at 25 °C, 150 rpm in 72 h incubation time. The present study involves one of the applications of pigments extracted from pigment producing microorganisms, in the colourisation of translucent candles and dyeing of paper.

Leifsonia aquatica, yellow pigment producing microorganism previously recognized as *Corynebacterium aquaticum leifson.* It was found that it has unusual chemotaxonomic characteristics such as 2,4 diaminobutyric (DAB) in the peptidoglycan and metaquinones of the MK 10 and MK 11. Afterwards it was separated from *Corynebacterium sp.* It has rarely been described as a cause of disease in humans. It is gram positive rods, motile and catalase positive and oxidase negative bacterium.

Pseudomonas aeruginosa is a common gram negative rod shaped bacterium. In certain conditions, *P. aeruginosa* can secrete a variety of pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown). The names pyocyanin and pyoverdine are from the Greek, with *pyo-*, meaning "pus", *cyanin*, meaning "blue", and *verdine*, meaning "green". Pyoverdine in the absence of pyocyanin is a fluorescent-yellow color. The most characteristic feature of *Pseudomonas aeruginosa* is the production of soluble pyocyanin pigment, a water soluble blue green phenazine compound which can be used as natural colourant in textile industry.

Organisms of the genus ***Kocuria*** (family Micrococcaceae, order Actinomycetales, class Actinobacteria) are gram positive coccoid bacteria often found as tetrads and irregular clusters. Colonies are pigmented giving orange shade in colour. Optimum growth temperature is 25-37 °C. Carotenoids are a class of hydrocarbons (carotenes) and their oxygenated derivatives are known as xanthophylls. Carotenoids are a class of fat soluble

pigments responsible for many of the red, orange, and yellow colour. It has novel characteristic is that it can degrade keratin.

Serratia marcescens, a gram-negative bacillus classified as a member of the Enterobacteriaceae. *S. marcescens* was considered originally to be an innocuous, non-pathogenic saprophytic water organism and was often used as a biological marker because of its easily recognized red colonies. Prodigiosin is a secondary metabolite of *Serratia marcescens* which is responsible for its red colour. This bacterium produces a bright red pigment when grown at temperatures below 35-37 $^{\circ}$C, but does not produce this pigment at higher temperatures, resulting in a pale off-white color. Perhaps it is noteworthy that the optimal growth range for *Serratia marcescens* is 25-37 $^{\circ}$C, and it is above this temperature range that pigment production ceases. It is possible that the loss of pigment is associated with mild temperature stress. Alternatively, it may be that temperature also plays a role in regulating the expression of the prodigiosin pathway.

Microbial pigments are suitable for mass production when compared to animal and plant extracts. There is an increasing interest involving bacteria as a possible alternate source of colourants used in foods, textile, pharmaceutical industries etc. Work carried out can be considered as innovative since we have tried to focus on application of pigments extracted from microorganisms to fast developing textile industry as a primary dyeing agent. Being in Surat city of Gujarat which have largest market share in Asia regarding manufacturing of Man Made Fabrics and processing, the concept of using microbial pigments will benefit humans and environment.

AIM

To obtain natural pigments from microbial source in economical viable process.

OBJECTIVES

- ✓ Isolation of pigment-producing bacteria.
- ✓ Designing growth medium by using waste materials.
- ✓ Optimization of fermentation conditions for pigment producing bacteria.
- ✓ Extraction of the pigments.
- ✓ Characterization of the extracted pigments.
- ✓ Antibacterial activity of the extracted pigments.
- ✓ To check the dye efficacy of characterised pigments for its ability to be used as dye on different fabrics.

METHODOLOGY

Isolation and Designing of Growth Medium:

Microorganisms which are capable to produce bio colours (pigments), were isolated from the soil sample collected from the campus of Veer Narmad South Gujarat University, Surat (Latitude: 21.1539871; Longitude: 72.7889031) and grown on nutrient agar plate (HiMedia, Mumbai).

From nutrient agar plate, isolated four chromogenic bacterial colonies of *Leifsonia aquatica*, *Pseudomonas aeruginosa*, *Kocuria rosea* and *Serratia marcescens* were further used for the production of considerable amount of yellow, green, orange and red pigment on the solid as well as in the liquid medium.

Selected strains were identified based on the morphological and biochemical characteristics, as described in Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974) as well as with the help of VITEK 2 system and ID-GNB card.

Production Medium & Fermentation Conditions:

Experiments were conducted in shake flask and pigment production was monitored after standardizing protocol. 50 mL of freshly prepared nutrient broth taken in 250 mL Erlenmeyer flask was inoculated directly with the pigment producers grown on nutrient agar plate. Flasks were incubated for 72 h on a rotary shaker at 150 rpm, at 25 ± 2°C temperature. 1 % glycerol was added for the enhancement of pigment production.

Extraction & Characterization of Microbial Pigments:

Maximal yield of pigment for extraction of culture broth was standardized using different solvents *viz*; methanol, ethyl acetate, petroleum ether, chloroform, diethyl ether and distilled water. Extraction of the pigment was done by the modified method given by P. Gunasekaran, 2005. After incubation, the grown cultures were centrifuged (REMI-laboratory Centrifuge, India) at 2200 rpm for 10 minutes. The coloured pellets were re-suspended in 95% methanol until pellets becomes colourless. Extracted pigments were filtered by Whatman filter paper no.1 (thickness 0.1 mm) and filtrates were concentrated by evaporation of the solvent. Evaporated extracted pigments were re-dissolved in ethyl acetate and methanol.

The coloured, redissolved pigments were then analyzed by U.V. Visible spectrophotometer (Shimadzu, Japan) for detecting their absorbance. The scanning range was selected 400-600 nm. Assumption tests were also done by crude extract to know the basic character of extracted pigment by modified method standardized in our laboratory. Extracted pigments were further analyzed by proton NMR and IR Spectrophotometer for determination of pigment characters.

Extracted pigments were separated by Thin Layer Chromatography (TLC). The solvent system used for the TLC was chloroform: methanol: acetic acid: water (90:8:1:0.8). Rf value of separated pigments was compared with standard values of pigments.

Antibacterial Potentiality:

The antibacterial activities were determined against various bacteria namely *Staphylococcus aureus*, *Salmonella paratyphi B*, *Escherichia coli* and *Bacillus subtilis*. The extracted yellow, green, orange and red pigments having definite concentration were used for to determine its antibacterial activity by disc diffusion and cup borer method (Experimental Microbiology, Patel R. J., 2011).

Application part of Extracted Pigments:

In Textile

The extracted pigments were subjected to effect of different temperature regimes, light intensities and effects of mordents (turpentine, lime juice, linseed oil) on its stability and staining properties if any to be used as dye to colour fabric.

Fastness testing after applying on different types of Fabric

-Extracted pigments were applied on different kinds of fabrics like cotton (natural), viscose (semi synthetic) and polyester (synthetic).

Colored fabrics were be also tested for -

- Washing fastness
- Perspiration fastness
- Light fastness
- Rubbing fastness

Yellow, red and orange pigments dyed the fabrics very nicely compared to green pigment. After checking the various fastness tests, colour pigments showed good stability on fabrics. Cotton fabric dyed good as compared to viscose and polyester.

In Candle making

Dyeing of candle was done using evaporated extracts of the pigments. Commercial translucent wax was placed in a beaker and heated until completely melted. Melted wax was mixed with evaporated extracts of pigments. After de-moulding, it was looked most similar to recently marketable coloured candles. So, it has potential to replace the synthetic colour from such products by natural colourants if production of such biocolours is made economical viable.

In Paper Printing

As both fabric and candle was successfully dyed with natural pigments, it can be applied in printing process or printing paper. White paper (Whatman filter paper no.1) was nicely dyed when it was filtered for further analysis. So, it is interpreted that for the paper dyeing, natural pigment may be used to replace synthetic dye in printing.

Conclusion

Microbial pigments are suitable for mass production when compared to animal and plant extracts. There is an increasing interest involving bacteria as a possible alternate source of colourants used in foods, textile, pharma etc. The yellow, green, orange and red-pigment producing bacteria from natural source showed significantly good application as a natural dye/biocolourant for colouring of cotton, viscose and polyester. The pigment also showed antibacterial property enhancing its business worth. Application of the natural pigment promotes consumers health protection and allows manufacturing of fully eco friendly pigment without any synthetic mix ultimately leading to better environment the place where all living organisms can stay in harmony.

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